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**Integrated genetic and morphological data support eco-evolutionary divergence of
Angolan and South African populations of *Diplodus hottentotus*.**

Running headline: Eco-evolutionary divergence in *Diplodus* spp.

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ABSTRACT

The *Diplodus* genus presents multiple cases of taxonomic conjecture. Among these the *D. cervinus* complex was previously described as comprising three subspecies that are now regarded as separate species: *D. cervinus*, *D. hottentotus* and *D. omanensis*. *Diplodus hottentotus* exhibits a clear break in its distribution around the Benguela Current system, prompting speculation that Angolan and South African populations flanking this area may be isolated and warrant formal taxonomic distinction. This study reports the first integrated genetic (mtDNA and nuclear microsatellite) and morphological (morphometric, meristic and colouration) study to assess patterns of divergence between populations in the two regions. High levels of cytonuclear divergence between the populations support a prolonged period of genetic isolation, with the sharing of only one mtDNA halotype (12 haplotypes were fully sorted between regions) attributed to retention of ancestral polymorphism. Fish from the two regions were significantly differentiated at a number of morphometric (69.5%) and meristic (46%) characters. In addition, Angolan and South African fish exhibited reciprocally diagnostic colouration patterns that were more similar to Mediterranean and Indian Ocean congeners, respectively. Based on the congruent genetic and phenotypic diversity we suggest that the use of '*hottentotus*', whether for full species or subspecies status, should be restricted to South African *D. "cervinus"* to reflect their status as a distinct 'species- like unit', while the relationship between Angolan and Atlantic/Mediterranean *D. "cervinus"* will require further demo-genetic analysis. This study highlights the utility of integrated genetic and morphological approaches to assess taxonomic diversity within the biogeographically dynamic Benguela Current region.

Key words: taxonomy; fish; morphometric; meristic; mitochondrial; microsatellite

INTRODUCTION

Within the family Sparidae there are 35 genera and 118 species described (Hanel & Tsigenopoulos, 2011). The genus *Diplodus* comprises 12 species, for which a number of sub-species have been described based on geographical differences and, often subtle, morphological variation (Hanel & Tsigenopoulos, 2011). While there is a general consensus relating to the taxonomy within the genus, Heemstra & Heemstra (2004) have suggested that many sub-species described around the Benguela Current system, a prominent marine biogeographic barrier, should be raised to full species status.

The *Diplodus cervinus* complex was previously described as comprising three subspecies: *Diplodus cervinus cervinus* (Mediterranean Sea and northeastern Atlantic Ocean), *D. c. hottentotus* (around southern Africa from Angola to Mozambique) and *D. c. omanensis* (Indian Ocean, endemic to Oman – see Figure I), but these taxa are now regarded as separate species (*D. cervinus*, *D. hottentotus* (Heemstra & Heemstra, 2004) and *D. omanensis* (Bauchot & Bianchi, 1984)). *Diplodus hottentotus* has a distinct break in its distribution, with no records of this species along the Namibian or South African west coast. It has been suggested that the southern Angolan and South African populations of *D. hottentotus* flanking this distribution break may be isolated by the cold water marine biogeographic barrier formed by the Benguela Current (Floeter *et al.*, 2008). Several studies have been conducted on the life history of *D. cervinus* from the Canary Islands (Pajuelo *et al.*, 2003a & b), Algeria (Derbal & Kara, 2006; 2010), South Africa (Mann & Buxton 1992, 1987, 1998),

and Angola (Winkler *et al.*, 2014 a,b). While there are significant differences between the life history parameters of the northern Atlantic & Mediterranean populations and the Angolan & South African populations, this could be due to sampling biases and the use of suspect aging and sexual pattern determination techniques. Moreover, there have been no taxonomic comparisons among Atlantic populations. As the Benguela Current system has been implicated as a major biogeographic barrier to gene flow and to be driving population-, sub-species-, and species-level divergences among marine fish in the region, empirical analysis of the eco-evolutionary relationship between Angolan and South African *D. hottentotus* is required.

The objective of this work was to explore the possible divergence between hitherto described conspecific Angolan and South African *D. hottentotus* populations. DNA barcoding using mitochondrial DNA (mtDNA) cytochrome oxidase I sequences (Hebert *et al.*, 2003) has been shown to be successful at identifying cryptic diversity among marine and freshwater taxa (Nwani *et al.*, 2011; Pereira *et al.*, 2013). However, inferences based on COI, or any single locus, may misrepresent a specie's/population's evolutionary history (Dupuis *et al.* 2012) and so genotyping of nuclear microsatellite loci was also performed here. As units identified through genetic patterns can be supported by divergence in morphological or biological traits (Thomas *et al.*, 2014) we also assess morphometric and meristic variation between populations from the two regions. Both genetic and morphological data reveal high levels of divergence between regional populations, which are interpreted along with other information for *D. cervinus* and *D. omanensis* in a taxonomic context.

MATERIALS & METHODS

GENETIC ANALYSIS

Sampling and DNA extraction

A total of 168 individuals of *D. hottentotus* were collected from thirteen sampling sites in Angola and South Africa, plus two outgroup individuals of *D. cervinus* from Turkey (see Figure 2 & Supplementary Table I). Samples were obtained from a mixture of recreational angling, spearfishing and local fish markets. A fin clip was removed from each individual and preserved in 95% ethanol. Total genomic DNA was extracted following the phenol-chloroform method described by Sambrook *et al.*, (1989) and visualised on a 1% agarose gel.

mtDNA sequencing and analysis

A 501bp fragment of the mtDNA cytochrome oxidase I (COI) gene was amplified using PCR with unpublished species-specific primers DCCOIF (5' TCATTCCGAGCCGAACTAAGC 3') and DCCOIR (5' TCCTGCAGGGTCAAAGAAAG 3'). PCRs comprised of 10 µl of BIOMIX (BioLine), 1.0 pMol of primer (both forward and

reverse), 6 µl of template DNA and 2 µl of sterile distilled water giving a total reaction volume of 20µl. All PCRs were performed using the following reaction conditions: 120 s at 95°C, then 40 cycles of 30 s at 94°C, 30 s at 50°C, 60 s at 72°C, with a final extension step of 120 s at 72°C. PCR amplicons were cleaned using SureClean (BioLine) and sequenced in both directions using Big Dye technology on an ABI 3730 DNA analyser (Applied Biosystems®). Sequence chromatograms were examined and edited in CHROMAS (Technelysium Ltd) and aligned using CLUSTAL W executed in BIOEDIT (Hall, 1999). Genetic variation was described using haplotype diversity (h , Nei and Tajima, 1981) with differentiation among samples quantified by Φ_{ST} (with significance assessed by 10 000 permutations) using ARLEQUIN 3.5 (Excoffier & Lischer, 2010). A median joining network was constructed in NETWORK (www.fluxus-engineering.com/sharenet.htm).

Microsatellite DNA genotyping and analysis

Following testing of 18 published nuclear microsatellite sparid loci a subset of seven polymorphic loci [DsaMS16, DsaM27, DsaMS34 (Perez et al., 2008), Dvul4, Dvul33, Dvul58, Dvul84 (Roques et al., 2007a,b)] which provided consistent PCR products were used to assess nuclear genetic variation among two samples from South Africa (Tsitsikamma and Port Elizabeth) and one sample from Angola (Flamingo). Loci were individually amplified by PCR using thermoprofiles consisting of 300s at 95°C, then 30 cycles of 30s at 92°C, 30s at a 55°C (but 50°C for Dvul33) and 30s at 72°C, and a final extension step of 72°C for 120s. All reactions used the following reaction mix: 5 µl of BIOMIX (BioLine), 0.5 pMol of primer (both forward and reverse), 3 µl of template DNA and 1 µl of sterile distilled

water giving a total reaction volume of 10 μ l. Alleles were separated using an AB3730 DNA analyser and allele identity inferred using Peak Scanner 2.

Numbers of alleles (N_A), allelic richness (A_R), observed heterozygosity (H_O), and expected heterozygosity (H_E), were calculated using FSTAT 2.9.3.2 (Goudet, 1995). Genotype frequency conformance at individual loci to Hardy-Weinberg equilibrium (HWE) expectations and genotypic linkage equilibrium between pairs of loci were tested using exact with default parameters in GENEPOP 3.3 (Raymond & Rousset, 1995). Multilocus values of significance for HWE tests were obtained using Fisher's method (Sokal and Rohlf, 1995) to combine probabilities of exact tests. The assumption of selective neutrality of the microsatellite loci was tested using the outlier method implemented in LOSITAN (Antao et al. 2008) following McKeown *et al.*, (2017). Genetic structuring without any prior information was investigated using the Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). Briefly, the analysis identifies the most probable number of genetically distinct groups (K) represented by the data and estimates assignment probabilities (Q) for each individual (specifically their genomic components) to these groups. Each MCMC run consisted of a burn in of 10^6 steps followed by 5×10^6 steps. Three replicates were conducted for each K to assess consistency. The K value best fitting the data set was estimated by the log probability of data [Pr(X/K)]. Clustering among individuals was also assessed using Discriminant Analysis of Principal Components (DAPC) implemented in ADEGENT (Jombart et al., 2010). Genetic differentiation among samples was also quantified by single- and multi-locus values of the unbiased F_{ST} estimator, θ (Weir and Cockerham, 1984), calculated using FSTAT, with the significance of estimates tested by 10 000

permutations of genotypes among samples (Goudet et al., 1996). F_{ST} values were also calculated employing the correction for potential null allele effects using FreeNA (Chapuis & Estoup, 2007)

MORPHOLOGICAL ANALYSIS

Sample collection, preservation and analysis

Fish were collected using spear fishing, hook-and-line, or purchased from local fish markets from Benguela, Lucira, Namibe, Flamingo Lodge and Tombua in southern Angola (n=25) and from Port Alfred, Port Elizabeth and Cape St Francis in South Africa (n=47). After capture, fish were sacrificed and immediately placed in 10% formalin. After at least one month, fish were transferred from the formalin to a 10% ethanol solution for three days, a 50% ethanol solution for three days, and final storage in a 70% ethanol solution.

Following preservation a total of 15 meristic counts and 47 morphometric measurements were made on each fish following Hubbs & Larger (1947) and Richardson (2011) and outlined in Supplementary Table II. All morphometric measurements were made using digital callipers to the nearest 0.01 mm. If a fish was damaged and a particular measurement was not possible, the measurement was estimated from a linear regression of

the form: $FL_i = mx_i + c$ where FL_i is the fork length of the damaged individual, m is the slope of the model and x_i is the missing character and c is the y intercept.

Since morphometric data are continuous and the meristic data are discrete, statistical analyses of both types were performed separately. Extreme outliers in the morphometric data from each region were defined as those greater than three times the inter-quartile range, below or above the first and third quartiles, and detected using a box plot analysis (Simon et al., 2010). Significant correlations between size (FL) and morphometric characters may accentuate such size differences (Simon et al., 2010) and complicate the morphometric comparisons. To eliminate this common problem associated with allometric growth variation, all morphometric measurements were size-adjusted to an overall mean fork length of 206.09 mm (the mean size of all samples) using the equation : $Y'_{ij} = \log Y_{ij} - b_j(\log FL_i - \log FL(\text{overall}))$ (Reimchen et al., 1985, Senar et al., 1994, Simon et al., 2010).

Differences between size-adjusted morphometric and meristic character means between Angolan and South African fish were tested using a two sample *t*-test. Both data sets were then analysed using a multi-dimensional scaling (MDS) incorporating the Bray-Curtis similarity measure. The extent of similarity between sites was assessed using a one-way analysis of similarity (ANOSIM) using the statistical package PAST Version 2.16 (Hammer et al., 2001) and were considered significant at $p < 0.05$.

RESULTS

GENETIC DIVERSITY

Pruning of mtDNA sequences permitted comparison of 501 sites across 96 individuals (Angola $n = 33$; South Africa $n = 40$; Turkey $n = 23$ [two sequences obtained here and 21 from GenBank]) and revealed a total of 13 haplotypes. Haplotype diversity was higher in the Angolan than South African sample (h (SD) = 0.73(0.06) and 0.36 (0.09) respectively) with an intermediate value for Turkey (h (SD) = 0.58 (0.088)). There was a clear phylogeographic partitioning of haplotypes between Angola and South Africa (Figure II) with only one haplotype (Haplotype 7) shared between these regions. Three haplotypes were identified among the Turkish samples and these were found to occupy central positions in the haplotype network with one (Haplotype 6) being the most common haplotype among South African samples, and the other two (Haplotypes 2 and 3) being the most common among the Angolan samples (Figure II). The clear partitioning of haplotypes between Angola and South Africa translated into large and highly significant Φ_{ST} (0.5; $P < 0.0001$). The Turkey sample also displayed significant Φ_{ST} values against Angola and South Africa, but with much lower values against Angola (0.06; $P < 0.05$) than South Africa (0.5; $P < 0.001$).

Information on microsatellite genetic variation for each sample / locus combination is provided in Supplementary Table III. There were no significant deviations from random associations of genotypes (linkage disequilibrium) detected for any pair of loci, either across all samples (data pooled) or in any single sample, indicating that all loci assort independently. No loci were identified as significant, putative non-neutral, outliers. All loci were variable in each sample with the total number of alleles per locus ranging from two (DsaMS27) to 28 (Dvul84) with an average of 8.43. Although levels of variability differed across loci, multi-

locus variability indices were similar across all samples. Significant deviations from HWE were found in 9 out of 21 locus / sample comparisons (Flamingo - 3 of 7 tests; Port Elizabeth - 3 of 7 tests; Tsitsikamma - 3 of 7 tests), in eight cases due to heterozygote deficits, whilst the Tsitsikamma / DsaMS34 comparison exhibited a heterozygosity excess. Bayesian clustering unanimously supported a model of $K = 2$ ($P = 1$ for $K = 2$, and zero for other models) with high assignment probabilities of all Flamingo (Angola) individuals to one cluster and Tsitsikamma and Port Elizabeth (South Africa) individuals to the other cluster (Figure III). This pattern was also evident following DAPC (Figure III). The pattern of genetic structuring between Angolan and South African samples was also supported by highly significant ($P < 0.0001$) pairwise F_{ST} values > 0.23 for comparisons between regions with similar values obtained after correction for null alleles. No significant differentiation was detected between Tsitsikamma and Port Elizabeth (F_{ST} without null allele correction = 0.019; with null allele correction = 0.017).

MORPHOLOGY

Only one individual from the morphometric dataset in the Angolan samples was identified as an extreme outlier and excluded from the subsequent analysis. The R^2 values for the linear regressions were all above 0.6 before transformation. These were however all below 0.05 after transformation, indicating that the transformed characters were free from a size bias. 32 of the 46 morphometric measurements were significantly different between South African and Angolan fish (Supplementary Table IV). The relationship between the most significant morphometric characters and fork length further provides evidence for

separation between the two regions (Figure IV). Seven of the 15 meristic counts also revealed significant differentiation between South African and Angolan fish (Supplementary Table V). The MDS ordination plot for both morphometric and meristic characters separated South African and Angolan individuals, with marginal overlap (Figure V). The ANOSIM results suggested a similar result to the MDS but also verified that the groupings were significantly different from one another ($P < 0.05$).

DISCUSSION

Combined analysis of genetic and morphological variation can provide synergistic insights into eco-evolutionary forces shaping biodiversity, as well as tools for conservation and management (Carriera et al., 2017). The present study represents the first integrated genetic and morphology based investigation within the *Diplodus* genus. A focus of this study was to assess evidence for divergence between conspecific populations of *D. hottentotus* in Angolan and South African waters. In line with *a priori* predictions, based on observations in other coastal fish species of evolutionary independence of populations across the Benguela Current system (Henriques, 2012; Henriques et al., 2012; Henriques et al., 2014; Henriques et al., 2016), high levels of genetic and morphological divergence between *D. hottentotus* populations in the two regions were found, which should prompt discussion of taxonomic revision in this species.

Congruent mtDNA and nuclear differentiation was observed between Angolan and South African samples of *D. hottentotus*, with a lack of differentiation within regions (though this could only be tested in South African waters). The mtDNA haplotype network, though shallow and with only five nucleotide differences between maximally diverged haplotypes, exhibited a clear phylogeographic structure: of 13 haplotypes resolved among South African and Angolan samples only one (haplotype 7, a tip haplotype) was found in both regions. This translated into high Φ_{ST} values between regions. Nuclear microsatellite variation also revealed a high level of differentiation between Angolan and South African samples which was supported by genetic clustering analyses. The strong assignment of individuals to their ‘regional’ clusters provided no evidence of migrants or first generation hybrids between regions. The cytonuclear differentiation between Angolan and South African samples therefore clearly supports the hypothesis of restricted gene flow and absence of dispersal across the Benguela Current

When applied to taxonomic questions genetic methods can avoid many of the pitfalls of assessments based only on morphology, but traditional mtDNA-based approaches have been criticised due to their over-reliance on strict exclusivity criteria such as reciprocal monophyly or barcoding gaps (reviewed in Sites & Marshall 2004; Hudson & Coyne 2002; Hudson & Turelli 2003; Moritz & Ciero, 2004). Specifically, mtDNA-based taxonomic inferences applying such strict criteria may be compromised by specimen misidentification, hybridisation and/or recent divergence (with the retention of ancestral polymorphism and incomplete lineage sorting). In the present study genetic and phenotypic alignment for all individuals excludes specimen misidentification, while patterns of nuclear differentiation provide no support for hybridisation or any recent introgression including male-biased gene

flow. In light of this, the sharing of haplotype 7 between Angolan and South African samples can be attributed to retention of ancestral polymorphism / incomplete lineage sorting. Even more compelling evidence of retention of ancestral polymorphism is provided by the presence of haplotype 6 (a central haplotype) in both the South African and Turkish samples but its absence from Angolan samples, and conversely the sharing of haplotypes 2 and 3 between Turkey and Angola but their absence from South Africa. Collectively the genetic patterns indicate considerable genetic divergence between Angolan and South African *D. hottentotus* but that insufficient time has passed for mtDNA variation to be completely sorted.

All three haplotypes identified in the Mediterranean are shared with, and are the common haplotypes among the African samples (two with Angola and one with South Africa). This pattern contrasts with results from a similar mtDNA analysis of other *Diplodus* species by Henriques (2012), who reported reciprocal monophyly of NE Atlantic *D. sargus* (formerly *D. sargus sargus*) and African *D. capensis* (formerly *D. sargus capensis*) with an estimated coalescence time of approximately 1.8 Ma. Similarly, Henriques (2012) reported a higher degree of mtDNA divergence between Angolan and South African samples of *D. capensis* than observed here for *D. hottentotus*. Coalescent depths among groups may vary considerably due to differences in population size, mutation rate and time since speciation (Monaghan *et al.*, 2009; Fujita *et al.*, 2012). Additionally, the faster generation time of *D. capensis* / *D. sargus* (sexual maturity at 1.8 years: Richardson *et al.*, 2011) compared to *D. hottentotus* / *D. cervinus* (sexual maturity at 4.9: Mann & Buxton, 1997) would permit faster lineage sorting in *D. capensis* / *D. sargus* in a given time even if other mutation/demographic processes were similar.

329

330 A high degree of phenotypic divergence between Angolan and South African *D.*
331 *hottentotus* was observed in morphometric ($R = 0.30$; significantly different mean values for
332 69% of characters) and meristic characters ($R = 0.42$; significantly different mean values for
333 46.1% of characters), and overall differentiation in the MDS ordination plots. Similar levels
334 of morphometric ($R = 0.34$) and meristic ($R = 0.35$) variation were reported between *D.*
335 *capensis* from Angola and South Africa (Richardson, 2011) however, despite the
336 aforementioned greater levels of genetic divergence fewer character means were
337 differentiated between both regions in that case. This indicates varying levels of plasticity /
338 adaptation and / or conservatism among these *Diplodus* species, which could compromise
339 taxonomic investigations based solely on phenotype. Plasticity and adaptation are also likely
340 to be key factors governing responses to future environmental change (King *et al.*, 2017).

341

342

343 Although general phenotype characteristics such as colouration are typically regarded
344 as highly plastic and of limited use as diagnostic characters, in the present study they do
345 reveal some intriguing macrogeographical patterns. As depicted in Figure I, Angolan
346 individuals were bronze in colour and lacked ventral abdominal stripes while those from
347 South Africa were more silver with intermittent belly stripes. Overall the Angolan colour
348 patterns were more similar to Mediterranean fish, while South African colour patterns were
349 more similar to fish from Oman. These phenotypic colouration patterns readily align with
350 those described previously by Bauchot & Bianchi (1984).

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The genetic differences among South African and Angolan samples are compatible with a prolonged period of population isolation and distinct evolutionary trajectories (Waples, 2008). The genetic diversity also aligns readily with regional differences in general phenotype and morphology. Such congruent genetic-morphological divergence has driven taxonomic reappraisals in other groups (e.g. Gobidae; Lima-filho *et al.*, 2016). With regard to the use of ‘hottentotus’, whether for full species or subspecies status, this should be restricted to South African *Diplodus “cervinus”* to reflect their status as distinct ‘species- like units’ (*sensu* Collins & Cruickshank 2013). Such a redefinition can be made conveniently due to the clear geographical separation of both units. The relationship between Angolan and Atlantic/Mediterranean *D. cervinus* will need to be further investigated through more extensive phenotypic and genetic sampling. The present study highlights that DNA barcoding has great value as an exploratory technique in taxonomy and for revealing cryptic diversity. However, it also shows that this potential can only be maximised if traditional COI-based approaches are complemented with data from other (independent) genetic loci, ontogenetic data and an appreciation of the limit of applying strict thresholds/exclusivity criteria. In light of the dynamics of speciation in the Benguela Current region, failure to do so or reliance on one method may compromise species delimitation and an underestimation of coastal African ichthyodiversity, thereby curtailing efforts to conserve evolutionarily distinct taxa in this complex marine system.

REFERENCES

Antao. T. Lopes, A. Lopes, R.J. Beja-Pereira, A. & Luikart, G. (2008). LOSITAN: A workbench to detect molecular adaptation based on a F_{9st}-outlier method. *BMC Bioinformatics* **9**. 323.

377 Bauchot, M.L. Bianchi, G. (1984). *Diplodus cervinus omanensis*, Nouvelle sous-espece de
 378 *Diplodus cervinus* (Lowe, 1941), capturée en mer d'Arabie (Pices Perciformes, Sparidae).
 379 *Cybium* **8**, 103:105.

380 Carreira, G. P. Shaw, P.W. Goncalves, J.M. & McKeown, N.J. (2017). Congruent molecular
 381 and morphological diversity of Macaronesian limpets: insights into eco-evolutionary forces
 382 and tools for conservation. *Frontiers in Marine Science*, **4**:75.

383 Chapuis, M. P. & Estoup, A. (2007). Microsatellite null alleles and estimation of population
 384 differentiation. *Molecular Biology and Evolution*, **24**(3): 621-631.

385 Collins, R. A. & Cruickshank, R. H. (2013). The seven deadly sins of DNA barcoding.
 386 *Molecular Ecology Resources*, **13**(6): 969-975.

387 Derbal, F. & Kara, M.H. (2006). Régime alimentaire du sar tambour, *Diplodus cervinus*
 388 *cervinus* (Sparidae) des côtes de l'est algérien. *Cybium*, **30**: 161-170.

389 Dupuis, J.R. Roe, A.D. & Sperling, F.A.H. (2012). Multi-locus species delimitation in closely
 390 related animals and fungi: one marker is not enough. *Molecular Ecology*, **21**(18): 4422-4436.

391 Excoffier, L. & Lischer, H. E. (2010). Arlequin suite ver 3.5: a new series of programs to
 392 perform population genetics analyses under Linux and Windows. *Molecular Ecology*
 393 *Resources*, **10**(3): 564-567.b

394 Floeter, S.R. Rocha, L.A. Robertson, D.R. Joyeux, J.C. Smith-Vaniz, W.F. Wirtz, P.
 395 Edwards, A.J. Barreiros, J.P. Ferreira, C.E.L. Gasparini, J.L. Brito, A. Falcó, J.M. Bowen,
 396 B.W. & Bernardi, G. (2008). Atlantic reef fish biogeography and evolution. *Journal of*
 397 *Biogeography*, **35**: 22-47.

398 Fujita, M. K., Leaché, A. D., Burbrink, F. T., McGuire, J. A. & Moritz, C. (2012) Coalescent-
 399 based species delimitation in an integrative taxonomy. *Trends in Ecology & Evolution*, 27(9):
 400 480-488.

401 Goudet, J. (1995). FSTAT (version 1.2): a computer program to calculate F-statistics. *Journal*
 402 *of Heredity*, **86(6)**: 485-486.

403 Goudet, J. Raymond, M. de Meeüs, T. & Rousset, F. (1996). Testing differentiation in diploid
 404 populations. *Genetics*, **144(4)**: 1933-1940

405 Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis
 406 program for Windows 95/98/NT. In *Nucleic acids symposium series* (Vol. 41), pp. 95-98.

407 Hammer, Ø. Harper, D.A.T. Ryan, P.D. (2001). PAST: Paleontological Statistics Software
 408 Package for Education and Data Analysis. *Palaeontologia Electronica* **4(1)**: 9pp.

409 Hanel, R. & Tsigenopoulos, C.S. (2011). Phylogeny, evolution and taxonomy of sparids with
 410 some notes on their ecology and biology. In: Pavlidis MA, Mylonas CC (eds.), *Sparidae:*
 411 *Biology and Aquaculture of the Gilthead Sea Bream and other species*. Sussex: Blackwell
 412 Publishing. pp 51-74.

413 Heemstra, P. & Heemstra, E. (2004). *Coastal fishes of Southern Africa*. South Africa:
 414 National Inquiry Service (NISC) and South African Institute of Aquatic Biodiversity
 415 (SAIAB).

416 Hebert, P. D. Stoeckle, M. Y. Zemlak, T. S. & Francis, C. M. (2004) Identification of birds
 417 through DNA barcodes. *PLoS Biology*, **2(10)**: p.e312.

418 Henriques, R. P. N. L. (2012). Influence of the Benguela Current in genetic substructuring of
 419 commercially exploited fish species. PhD thesis, Royal Holloway, University of London.

420 Henriques, R. Potts, W. M. Sauer, W. H. H. & Shaw, P. W. (2012) Evidence of deep genetic
 421 divergence between populations of an important recreational fishery species, *Lichia amia* L.
 422 1758, around southern Africa. *African Journal of Marine Science* **34(4)**: 585-591.

423 Henriques, R. Potts, W. M. Santos, C. V. Sauer, W. H. & Shaw, P. W. (2014) Population
 424 connectivity and phylogeography of a coastal fish, *Atractoscion aequidens* (Sciaenidae),
 425 across the Benguela Current Region: evidence of an ancient vicariant event. *PloS one* **9(2)**:
 426 p.e87907.

427 Henriques, R. Potts, W. M. Sauer, W. H. Santos, C. V. Kruger, J. Thomas, J. A. & Shaw, P.
 428 W. (2016) Molecular genetic, life- history and morphological variation in a coastal warm-
 429 temperate sciaenid fish: evidence for an upwelling- driven speciation event. *Journal of*
 430 *Biogeography* **43(9)**: 1820-1831.

431 Hubbs, C.L. & Lagler, K.F. (1947). Fishes of the great lakes region. *Bulletin of the*
 432 *Cranbrook Institute of Science*, **26**: 8-21.

433 Hutchings, K. Griffiths, M.H. & Field, J.G. (2006). Regional variation in the life history of
 434 the canary drum *Umbrina canariensis* (Sciaenidae), in South African waters. *Fisheries*
 435 *Research*, **77**: 312-325.

436 Hudson, R. R. & Coyne, J. A. (2002). Mathematical consequences of the genealogical species
 437 concept. *Evolution*, **56(8)**: 1557-1565.

438 Hudson, R. R. & Turelli, M. (2003) Stochasticity overrules the “three- times rule”: genetic
 439 drift, genetic draft, and coalescence times for nuclear loci versus mitochondrial DNA.
 440 *Evolution*, **57(1)**: 182-190.

441 Jombart, T. Devillard, S. & Balloux, F. (2010). Discriminant analysis of principal
 442 component: a new method for the analysis of genetically structure populations. *BMC*
 443 *Genetics*, **11**: 94.

444 King, N.G. McKeown, N.J. Smale, D.A. & Moore, P.J. (2017). The importance of phenotypic
 445 plasticity and local adaptation on driving intraspecific variability in thermal niches of marine
 446 macrophytes. *Ecography*, **40**:01-014.

447 Limo-Filho, P.A. de Souza Rosa, R. se Santos de Souza, A. da Costa G.W.W.F. de Oliveira,
 448 C. & Molina, W.F. (2016) Evolutionary dversification of western Atlantic *bathygobius*
 449 soecies based on cytogenetic, morphologic and DNA barcode data. *Reviews in Fish Biology*
 450 *and Fisheries*, **26**: 109-121.

451 Mann, B.Q. & Buxton, C.D. (1992). Diets of *Diplodus sargus capensis* and *D. cervinus*
 452 *hottentotus* (Pices: Sparidae) on the Tsitsikamma coast, South Africa. *Koedoe*, **35**: 27-32.

453 Mann, B.Q. & Buxton, C.D. (1997). Age and growth of *Diplodus sargus capensis* and *D.*
 454 *cervinus hottentotus* (Sparidae) on the Tsitsiskamma coast, South Africa. *Cybiurn*, **21**: 135-
 455 147.

456 Mann, B.Q. & Buxton, C.D. (1998). Reproductive biology of *Diplodus sargus capensis* and
 457 *D. cervinus hottentotus* (Sparidae) off the south-east Cape coast, South Africa. *Cybiurn*, **22**:
 458 31-47.

459 McKeown, N.J. Hauser, L. & Shaw, P.W. (2017). Microsatellite genotyping of brown crab
 460 reveals fine scale selection and ‘non-chaotic’ genetic patchiness within a high gene flow
 461 system. *Marine Ecology Progress Series*, **566**: 91-103.

462 Monaghan, M. T. Wild, R. Elliot, M. Fujisawa, T. Balke, M. Inward, D. J. Lees, D. C.
 463 Ranaivosolo, R. Eggleton, P. Barraclough, T. G. & Vogler, A. P. (2009) Accelerated species

464 inventory on Madagascar using coalescent-based models of species delineation. *Systematic*
 465 *Biology*: p.syp027.

466 Moritz, C. & Cicero, C. (2004) DNA barcoding: promise and pitfalls. *PLoS Biol*, **2(10)**:
 467 p.e354.

468 Nei, M. & Tajima, F. (1981). DNA polymorphism detectable by restriction endonucleases.
 469 *Genetics*, **97**: 145–163.

470 Nwani, C. D. Becker, S. Braid, H. E. Ude, E. F. Okogwu, O. I. & Hanner, R. (2011) DNA
 471 barcoding discriminates freshwater fishes from southeastern Nigeria and provides river
 472 system-level phylogeographic resolution within some species. *Mitochondrial DNA*, **22(sup1)**:
 473 43-51.

474 Pajuelo, J.G. Lorenzo, J.M. Dominguez-Seoane, R. (2003a). Age estimation and growth of
 475 the zebra seabream *Diplodus cervinus cervinus* (Lowe, 1838) on the Canary Islands shelf
 476 (Central-east Atlantic). *Fisheries Research*, **62**: 97-103.

477 Pajuelo, J.G. Lorenzo, J.M. Dominguez-Seoane, R. Ramos, A. & Gregoire, M. (2003)b. On
 478 population ecology of the zebra seabream *Diplodus cervinus cervinus* (Lowe, 1838) from the
 479 coast of the Canarian archipelago, North West Africa. *Environmental Biology of Fish*, **67**:
 480 407-416.

481 Pereira, L.H. Hanner, R. Foresti, F. & Oliveira, C. (2013). Can DNA barcoding accurately
 482 discriminate megadiverse Neotropical freshwater fish fauna? *BMC genetics*, **14**:20.

483 Perez, L. Infante, C. Ponce, M. Crespo, A. Zuasti, E. Funes, V. Catanese, G. & Manchado,
 484 M. (2008). Characterization of eight microsatellite markers in the white sea bream, *Diplodus*
 485 *sargus* (Teleostei, Sparidae). *Molecular Ecology Resources*, **8(6)**: 1291-1293.

486 Pritchard, J. K. Stephens, M. & Donnelly, P. (2000) Inference of population structure using
 487 multilocus genotype data. *Genetics*, **155**(2): 945-959.

488 Raymond, M. & Rousset, F. (1995) GENEPOP (version 1.2): population genetics software
 489 for exact tests and ecumenicism. *Journal of Heredity*, **86**: 248-249

490 Reimchen T.E. Stinson E.M. & Nelson J.S. (1985). Multivariate differentiation of parapatric
 491 and allopatric populations of threespine stickleback in the Sangan River watershed, Queen
 492 Charlotte Islands. *Canadian Journal of Zoology*, **63**: 2944-2951.

493 Richardson T.J. (2011). The Taxonomy, Life History and Population Dynamics of Blacktail,
 494 *Diplodus Capensis* (Perciformes: Sparidae), in southern Angola. MSc Thesis, Rhodes
 495 University, South Africa.

496 Richardson T.J. Potts, W.M. & Sauer W.H.H. (2011c). The reproductive style of *Diplodus*
 497 *capensis* (Sparidae) in southern Angola: rudimentary hermaphroditism or partial protandry?
 498 *African Journal of Marine Science*, **33**: 321-326.

499 Roques, S. Galarza, J. A. Macpherson, E. Turner, G. F. & Rico, C. (2007a) Isolation and
 500 characterization of nine polymorphic microsatellite markers in the two- banded sea bream
 501 (*Diplodus vulgaris*) and cross- species amplification in the white sea bream (*Diplodus*
 502 *sargus*) and the saddled bream (*Oblada melanura*). *Molecular Ecology Notes*, **7**(4): 661-663.

503 Roques, S. Galarza, J. A. Macpherson, E. Turner, G. F. Carreras-Carbonell, J. & Rico, C.
 504 (2007b) Isolation of eight microsatellites loci from the saddled bream, *Oblada melanura* and
 505 cross-species amplification in two sea bream species of the genus *Diplodus*. *Conservation*
 506 *Genetics*, **8**(5): 1255-1257.

507 Sambrook, J. Fritsch, E. F. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual.
 508 Cold Spring Harbor Press, Cold Spring Harbor, New York

509 Senar, J.C. Leonart, J. & Metcalfe, N.B. (1994). Wing-shape variation between resident and
 510 transient wintering Siskins *Carduelis spinus*. *Journal of Avian Biology*, **25**: 50-54.

511 Simon, K.D. Bakar, Y. Temple, S.E. & Mazlan, A.G. (2010). Morphometric and meristic
 512 variation in two congeneric archer fishes *Toxotes chatareus* (Hamilton 1822) and *Toxotes*
 513 *jaculatrix* (Pallas 1767) inhabiting Malaysian coastal waters. *Biomedicine and Biotechnology*,
 514 **11**: 871-879.

515 Sites Jr, J. W. & Marshall, J. C. (2004) Operational criteria for delimiting species. Annual
 516 Review of Ecology, Evolution, and Systematics: 199-227.

517 Sokal, R.R. & Rohlf, F.J. (1995) Biometry: the principles and practise of statistics in
 518 biological research. 3rd Edition.

519 Thomas, R.C. Willette, D.A. Carpenter, K.E. & Santos, M.D. (2014) Hidden diversity in
 520 sardines: genetic and morphological evidence of cryptic species in the goldstripe sardinella,
 521 *Sardinella gibbosa* (Bleeker, 1849). *PloS ONE* **9**(1):e84719.

522 Waples, R. S. (1998) Separating the wheat from the chaff: patterns of genetic differentiation
 523 in high gene flow species. *Journal of Heredity*, **89**: 438-450.

524 Weir, B. S. Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population
 525 structure. *Evolution*, **38**(6):1358-1370.

526 Winkler, A. C. Santos, C. V. & Potts, W. M. (2014a). Ontogenetic and seasonal shifts in the
 527 diet of *Diplodus cervinus hottentotus* (Pisces: Sparidae) in southern Angola. *African Journal*
 528 *of Marine Science*, **36**(3): 323-330.

529 Winkler, A. C. Santos, C. V. & Potts, W. M. (2014b). Diagnosing the sexual pattern of
530 *Diplodus cervinus hottentotus* (Pisces: Sparidae) from southern Angola. *African Journal of*
531 *Marine Science*, **36**(4): 505-512.